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13. ABSTRACT (Maximum 200 Words)

Breast cancers are often characterized by increased growth factor signaling pathways and numerous cell cycle alterations. PR are phosphorylated by CDK2 *in vitro* and *in vivo* at multiple sites including serine 400 (Ser400). The purpose of these studies is to investigate the role that growth factors and cell cycle molecules play on the regulation of PR by phosphorylation of Ser400. Treatment of T47D breast cancer cells with mitogens increased the phosphorylation of PR Ser400, as did the synthetic progestin R5020. Progestin dependent phosphorylation of Ser400 was reversed by a CDK2 inhibitor. Overexpression of cyclin E and CDK2 resulted in downregulation of PR protein in the absence of ligand. This effect was blocked by a CDK2 inhibitor. P27 is a cyclin-dependent kinase inhibitory protein. A p27-/- cell line was used to measure the transcriptional activity of PR following transient co-transfection of PR and a progesterone responsive-element. Ligand-independent PR transcriptional activity was elevated in p27-/- cells; mutation of PR serine 400 to alanine resulted in loss of PR transcriptional activity. In addition, cyclin E and CDK2 associated with wt PR in co-immunoprecipitation experiments. Regulation of PR by altered cyclin/CDKs may confer a selective advantage to breast cancer cells.

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Introduction:
Steroid hormones are required for normal breast development and play a key role in breast cancer. The steroid hormone progesterone regulates cell growth in the normal mammary gland and uterus by cell cycle phase -specific actions. Breast cancers are often characterized by increased growth factor signaling pathways and numerous cell cycle alterations, including decreased levels of p27 and increased levels of cyclins D1, D2 and E. Progestins, via the activation of progesterone receptor (PR), activate cyclin dependent kinase 2 (CDK2) and raise levels of cyclins D and E. PR are phosphorylated by CDK2 *in vitro* and *in vivo* at multiple sites including serine 400 (Ser400). In addition, breast cancer cell growth is controlled, in part by, cross-talk between steroid hormone and growth factor signaling pathways. The purpose of these studies is to investigate the role that growth factors and cell cycle molecules play on the regulation of PR by phosphorylation of Ser400.

STATEMENT OF WORK

Specific Aim 1: Determine effects of cyclin E and CDK2 overexpression on PR-B expression.

Task 1: Months 1-4. Measure PR protein levels following transient co-expression of either wild-type or S400A mutant PR in HeLa cell line with HA-tagged cyclin E and myc-tagged Cdk2 or empty control vector. *PR levels were found to be altered in the presence of active CDK2 compared to kinase dead CDK2.*

Task 2: Months 4-12. Measure PR protein levels following transient expression of Ad-cyclin E and Ad-CDK2 or adenoviral-GFP control in the T47D-YB breast cancer cell line.
The virus expression time course took too long to accomplish our goal of looking at PR expression patterns. Determining that the virus was not going to work was a good learning process for me. I had to develop an alternative direction. I designed an antibody that specifically recognizes the phosphorylated form of S400 to investigate the regulation of this site in cells that endogenously express PR. I found that this site is highly regulated by mitogens such as FBS and EGF in addition to ligand.

Task 3: Months 12-18.Measure PR protein levels following transient expression of Ad-cyclin E and Ad-CDK2 or Ad-GFP into MCF7 and T47D cell lines stably expressing either Wt or S400A mutant PR. *Not doing viral transfections to overexpress cyclin E or CDK2.*

Specific Aim 2: Determine effects of cyclin E and CDK2 overexpression on PR-B transcriptional activity.

Task1: Months 18-24. Measure PR transcriptional activity following co-transfection of either WT or mutant PR and HA-cyclin E, Myc-CDK2 and PRE- luciferase reporter gene in HeLa cells. *PR expression was determined to be altered in the presence of overexpressed cyclin E and CDK2. In addition, PR activity was altered in p27-/- cells obtained from Robert Sheaff (U of MN). Serine 400 was found to be required for this activity.*

Task 2: Months 24-30. Measure PR transcriptional activity following co-transfection of Ad-cyclin E, Ad-CDK2 and Ad-PRE- luciferase reporter gene in MCF7 cells stably expressing

either WT or S400A mutant PR. *Not doing viral transfections to overexpress cyclin E or CDK2.*

Task 3: Months 28-30. Measure expression of endogenous genes known to be regulated by progestins (i.e. c-myc, IRS-1) in stable cells lines expressing either Wt or mutant PR. *Not doing viral transfections to overexpress cyclin E or CDK2.*

Specific Aim 3: Determine effects of cyclin E and CDK2 overexpression on cell growth.

Task 1: Months 1-6. Stably transfect MCF7 and T47D breast cancer cell lines with S400A mutant PR. Note: We already have cells expressing WT PR. *S400A PR is expressed normally when transiently expressed. However, when stably expressed, S400A PR appears to give the cells a growth disadvantage. I have tried numerous times and the cells express a truncated gene product. I am in contact with some people in the field to determine what this means. It appears that Serine 400 may be at a protease cut site. In addition, time course studies with the phospho-400 antibody indicate that PR phosphorylated at this site are a more stable form of PR. An alternative will be to make cells that have inducible expression of PR.*

Task 2: Months 30-36.Measure effects on cell growth following addition of Ad-cyclin E, Ad-CDK2 in MCF7 cells stably expressing either WT or S400A mutant PR by flow cytometry and FACS analysis of propidium iodine-stained nuclei. *Have not been able to do without stable cell lines.*

Additional work:
I did immunoprecipitation (IP) experiments in T47D-YB cells with endogenous PR. These studies indicate that PR is in a complex with cyclin E and CDK2 in the absence of ligand. Future studies will determine the role(s) for either CDK2 complex formation or CDK2 activity leading to the regulation of PR. These studies have already begun. In general, I spent about a year trying to get the viral expression system working before deciding it was not going to answer the question I was asking. I have learned a lot about how to find different methods that can be used to answer a single question. These studies are currently going very well.

Key Accomplishments:

- Determined that Serine 400 is highly regulated by ligand and mitogens.
- Verified that Serine 400 is phosphorylated by CDK2.
- Determined that phospho-ser400 forms of PR are more stable than total PR.
- Determined that there is an increase in PR activity when CDK2 is no longer regulated by p27-/-.
- Determined that Serine 400 plays a role in regulating PR activity in p27-/- cells.
- Determined that CDK2 activity regulates PR transiently expressed in HeLa cells.
- Determined that cyclin E, CDK2 and PR form a complex.

Reportable outcomes:

- I presented my data in an oral session at the 2003 Endocrine Society's 85th Annual Meeting.
- 2003 Award Recipient (1st place) for Department of Medicine Research Day.
- 2003 Louise M. Nutter Student Research Award

- A manuscript and a book chapter are in preparation.

Conclusions: In general, I spent about a year trying to get the viral expression system working before deciding it was not going to answer the question I was asking. I have learned a lot about how to find different methods that can be used to answer a single question. My studies are currently going very well.